

FORM PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 550-128
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/284009 (To Be Assigned)
INTERNATIONAL APPLICATION NO. PCT/GB97/02709	INTERNATIONAL FILING DATE 8 October 1997	PRIORITY DATE CLAIMED 9 October 1996 30 January 1997 21 February 1997
TITLE OF INVENTION MONONUCLEAR PHAGOCYTES IN THERAPEUTIC DRUG DELIVERY		
APPLICANT(S) FOR DO/EO/US LEWIS et al		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.		
2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.		
3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).		
4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19 th month from the earliest claimed priority date.		
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).		
a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).		
6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).		
7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).		
a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made.		
8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).		
9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).		
10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
Items 11. To 16. Below concern document(s) or information included:		
11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.		
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.		
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.		
14. <input type="checkbox"/> A substitute specification.		
15. <input type="checkbox"/> A change of power of attorney and/or address letter.		
16. <input checked="" type="checkbox"/> Other items or information. Copies of International Search Report and International Preliminary Examination Report		

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) (To Be Assigned)		INTERNATIONAL APPLICATION NO. PCT/GB97/02709		ATTORNEY'S DOCKET NUMBER 550-128	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5): -- Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00 -- International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00 -- International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$760.00 -- International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00 -- International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 840.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 0.00	
CLAIMS		NUMBER FILED		NUMBER EXTRA	
Total Claims		24		-20 = 4	
Independent Claims		6		-3 = 3	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				+\$260.00	
TOTAL OF ABOVE CALCULATIONS =				\$ 1146.00	
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				0.00	
SUBTOTAL =				\$ 1146.00	
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				0.00	
TOTAL NATIONAL FEE =				\$ 1146.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				0.00	
Fee for Petition to Revive Unintentionally Abandoned Application (\$1,210 - Small Entity Fee = \$605)				0.00	
TOTAL FEES ENCLOSED =				\$ 1146.00	
				Amount to be: refunded \$	
				charged \$	
a. <input checked="" type="checkbox"/> A check in the amount of \$1146.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 14-1140 in the amount of \$_____ to cover the above fees. A duplicate copy of this form is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: NIXON & VANDERHYE P.C. 1100 North Glebe Road, 8 th Floor Arlington, Virginia 22201 Telephone: (703) 816-4000					
				SIGNATURE B.J. Sadoff NAME	
				36,663 April 5, 1999 REGISTRATION NUMBER Date	

09/284009

510 Rec'd PCT/PTO 05 APR 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

LEWIS et al

Atty. Ref.: **550-128**

Serial No. **(To Be Assigned)**

Group:

Filed: **April 5, 1999**

Examiner:

For: **MONONUCLEAR PHAGOCYTES IN THERAPEUTIC
DRUG DELIVERY**

* * * * *

April 5, 1999

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

IN THE CLAIMS

Claims 3, 5, 6, 13 and 14, line 1 of each, delete "any preceding" and after "Claim" insert -- 1 --.

Claim 8, line 1, delete "Claims 1-7" and insert -- Claim 1 --.

Claim 11, line 1, delete "Claims 1-6" and insert -- Claim 1 --.

Claim 12, line 1, delete "Claims 3 to 11" and insert -- Claim 1 --.

Claim 16, line 1, delete Claims 3 to 15" and insert -- Claim 1 --.

Claim 18, line 1, delete "or 17".

Claim 23, line 3, delete "Claims 1 to 18" and insert -- Claim 1 --.

LEWIS et al
Serial No. (To Be Assigned)

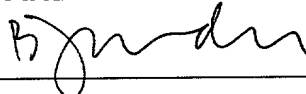
REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By:



B.J. Sadoff

Reg. No. 36,663

BJS:Imy

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

MONONUCLEAR PHAGOCYTES IN THERAPEUTIC DRUG
DELIVERY

The invention relates to a method of drug delivery; means therefor including components thereof which have particular, but not exclusive, application in
5 cancer therapy development.

Macrophages often comprise 20-60% of the tumour cell mass in breast carcinomas and form intimate contacts with malignant cells. This has long been thought to represent part of the host's defence mechanisms against the tumour; however, their function at such sites in the body remains an enigma at present
10 as macrophages isolated from human or murine tumours exhibit reduced tumouricidal and antigen-presenting activities compared to those from normal tissues (1).

Monocytes are produced in the bloodstream and extravasate (i.e. exit) into surrounding tissues including such diseased tissues as malignant tumours and
15 atherosclerotic plaques, where they differentiate into macrophages and perform immune, secretory, phagocytic and other functions. Monocytes and macrophages are collectively termed mononuclear phagocytes. As tissue macrophages have a lifespan of 60 to 90 days and the number of macrophages in tumours remains constant, it is believed that there is a constant attachment of
20 monocytes to the tumour endothelium and influx of monocytes into the tumour cell mass.

Hypoxia, that is, very low levels of oxygen, exist only in some forms of diseased tissue (e.g. malignant tumours, ischaemic heart tissue etc). Hypoxia and/or hypoglycaemia is thought to occur in growing tumours when the increasing metabolic demands of the rapidly expanding tumour cell population outstrip the supply of oxygen/glucose etc., made available to them by simple diffusion across the tumour mass from vessels in surrounding normal tissues.

Ischaemia, that is, a deficiency of blood flow to part of the body also exists in some forms of diseased tissue (e.g. malignant tumours, ischaemic heart tissue etc). Malignant tumours tend to outgrow their blood supply and often have ischaemic areas of tumour cell apoptosis and necrosis. Moreover, the vascular supply in some tumour areas can collapse resulting in the formation of further ischaemic areas. Ischaemic tissues are also found in coronary artery disease, diabetic retinopathy and following strokes. Cells in such areas of vascular occlusion or collapse experience various forms of stress such as glucose deprivation, low pH, elevated levels of lactate and pyruvate, ion or cationic imbalance and hypoxia.

Our recent and surprising data indicate that once monocytes enter a tumour from the bloodstream and differentiate into macrophages, they preferentially congregate in hypoxic (i.e. poorly vascularised and necrotic) sites deep within a tumour mass remote from blood vessels (2). Refer to Figure 1, which represents a bar chart of the Distribution of Macrophages in Relation to Blood Vessels. Moreover, breast tumours, with more hypoxic/necrotic areas, are more heavily infiltrated with macrophages, which preferentially locate to, or around, the necrotic sites (refer to Figure 2, which represents a bar chart of the Association of Macrophage Index with Necrosis in Breast Carcinomas). Very

recently, macrophages have also been shown to congregate in ischaemic/necrotic sites in ovarian tumours (3). Over a decade ago experimental hypoxia was shown to induce the production of angiogenic factors by macrophages *in vitro* (2). Taken together these data could underpin our recent finding, that increased numbers of macrophages in breast tumours equate with higher levels of angiogenesis and increased fatalities in breast cancer (2).

We examined tumour biopsies from 100 breast cancer patients, and found that macrophage infiltration was strongly associated with reduced relapse-free interval and overall survival - even in lymph node negative patients alone (i.e. the better prognosis group). Indeed macrophage infiltration proved to be almost as important in predicting outcome as lymph node status in breast cancer. We went on to see if this was due to an effect of macrophages on such important parameters of tumour aggressiveness as the mitotic index, receptor status, degree of angiogenesis, etc. It was at this point that a highly significant and unexpected correlation between the degree of macrophage infiltration within a tumour mass and angiogenesis emerged, suggesting a role for this cell type in some or all of the steps of tumour angiogenesis (2).

Our unique observations suggest that, since the entry or presence of macrophages into or in such diseased tissues appears to be deleterious to the patient, therapies specifically focused on either blocking their entry; their destruction; and/or their exploitation to carry therapeutic agents into such diseased tissues could prove to have therapeutic benefits.

Monoclonal antibodies have been considered for many years to be the best way of delivering cytotoxic agents to tumours, but this approach has so far been

disappointing in clinical trials (4, 5). The main reason for the lack of therapeutic efficacy of antibody conjugates in solid tumours is the resistance of the tumour to penetration by macromolecules. In studies with radiolabelled antibodies, typically only 0.001-0.01% of the injected dose localises to each gram of solid tumours in humans (6, 7). The poor penetration of antibodies is thought to be due to a number of factors. Firstly, the antibodies must cross the physical barrier of the endothelial cell layer in tumour blood vessels and the, often dense, fibrous stroma packed between tumour cell areas. Secondly, the dense packing of tumour cells and tight junctions between epithelial tumour cells hinder the transport of the antibody within the tumour mass. Thirdly, the absence of lymphatics within the tumour contributes to the build up of a high interstitial pressure which opposes the influx of molecules into the tumour core.

One solution to the poor penetration of antibody conjugates into solid tumours would be to attack the endothelial cells in the tumour, instead of the tumour cell themselves, which are readily accessible to intravenously injected antibody.

This then leads to destruction of the tumour blood vessels and the death of neighbouring tumour cells which rely on the blood supply for oxygen and nutrients. Early studies using murine models have met with some success in this area (8), but there remains the problem in humans of how to target tumour endothelial cells and not those in normal tissues. Furthermore, as mentioned earlier, our work strongly suggests the tumour necrosis resulting from this approach will trigger compensatory angiogenic activities in tumour-infiltrating macrophages in the vicinity. This would oppose the effects of the therapeutic agent.

Our inventive solution is to attach the agent (e.g. tumour or endothelial cell

cytotoxin) to monocytes in the peripheral blood, that is the cells which gain entry to the tumour in large numbers to form tumour-infiltrating macrophages.

Since monocytes swarm to the tumour site in large numbers as an early and ongoing event in tumour development, they can be used to carry therapeutic agents (e.g. cytotoxic drugs or toxins) into the centre of solid tumours. In support of this suggestion is the fact that many studies have shown that malignant tumours actively recruit this cell type and that monocytes then follow a chemotactic trail produced by tumour cells. Further, we have uniquely shown that mononuclear phagocytes congregate in tumour areas where they are most needed, i.e. hypoxic or stress areas, possibly to help initiate angiogenesis.

Although we have described the invention with particular reference to tumour cells, it can be used in any instance where mononuclear phagocytes infiltrate or are attracted to hypoxic/ischaemic/stress tissue or conditions. Thus, the invention can be used during development to control the vascularisation of developing tissue, typically, but not exclusively, with a view to targeting a hypoxia regulatable agent so as to promote or enhance vascularisation. Alternatively the invention can be used to target hypoxia regulatable agents to damaged tissue, for example to tissue where de-vascularisation has occurred following damage to the vascular system via an amputation, stroke, cardiac arrest, extreme hypertension, ischaemia, burns etc.

It follows from the information provided herein that the invention may be used to prevent or reduce tissue vascularisation, or to promote or enhance vascularisation, or to simply deliver selected drugs to hypoxic/ischaemic/stress sites where mononuclear phagocytes are typically present.

In addition, given our finding that mononuclear phagocytes infiltrate or are attracted to hypoxic/ischaemic/stress tissue or conditions the invention can also be used with a view to targeting a stress regulatable agent such as an ischaemically regulatable agent. Thus, in the instance where mononuclear phagocytes infiltrate a site which is also ischaemic and thus characterised by glucose deprivation, low pH, elevated levels of lactate or pyruvate, and/or hypoxia it is possible to arrange for targeting of an ischaemically regulatable agent.

It is therefore an object of the invention to provide a novel drug delivery system which exploits the fact that mononuclear phagocytes collect or are attracted to hypoxic or ischaemic or stress sites.

It is yet a further object of the invention to provide a regulatable drug delivery system which enables the activation of said drug to be controlled, and more specifically, to be controlled so as to only be active under hypoxic and/or stress and/or ischaemic conditions.

It is yet a further object of the invention to provide a novel drug for use in the drug delivery system of the invention.

The invention, in its broadest aspect, comprises the use of mononuclear phagocytes to deliver therapeutic agents to tissues and especially hypoxic and/or stress and/or ischaemic sites.

According to a first aspect of the invention there is therefore provided a therapeutic composition comprising a regulatable agent and an agent that binds

to a cell surface element of a mononuclear phagocyte.

In a preferred embodiment of the invention said binding agent preferentially binds to said cell surface element.

Reference herein to the term preferentially is intended to include the selective
5 or targeted binding of said agent to mononuclear phagocytes.

In a preferred embodiment said agent is a hypoxia and/or ischaemia and/or stress regulatable agent.

It will therefore be apparent that the hypoxia and/or ischaemia and/or stress regulatable agent will be affected by hypoxic and/or ischaemic and/or stress
10 conditions and typically affected so as to only be active in such conditions. Moreover, said binding agent, which is typically coupled to said regulatable agent, attaches the composition to mononuclear phagocytes and so targets the regulatable agent, to sites typically infiltrated by mononuclear phagocytes. Thus
15 in the instance where said mononuclear phagocytes penetrate hypoxic and/or ischaemic and/or stress sites said composition is suitably delivered to such sites and the regulatable agent becomes active.

The invention is elegant in so far as the body's own mechanisms are exploited for the specific delivery of drugs but the invention is safe in so far as the drugs remain inactive until exposed to hypoxic and/or ischaemic and/or stress
20 conditions.

Given the above nature of the invention agents suitable for use in manufacturing

the said composition will be known to those skilled in the art and therefore the following preferred embodiments are not intended to be exhaustive but rather illustrative.

For example, in one embodiment of the invention said hypoxia regulatable agent
5 may comprise a therapeutic gene, that is to say a nucleotide sequence encoding a therapeutic agent which is under the control of a hypoxia sensitive agent such as a hypoxia regulated expression element i.e. a promoter or enhancer which is sensitive to hypoxia. Thus, under conditions of hypoxia said element will be
10 expressed. In this embodiment of the invention the binding agent comprises an agent that ensures internalisation of said therapeutic gene, for example, without limitation a viral or a non-viral vector, with a view to incorporating same into the mononuclear phagocyte genome.

In addition, or alternatively, the hypoxia regulatable therapeutic gene may
15 encode a prodrug activation enzyme, that is to say an enzyme which converts a relatively inactive drug into a more active one. An example of this kind of enzyme is thymidine phosphorylase which activates the 5-FU prodrugs capcetabine and furtulon. Another example of a prodrug activation enzyme is the herpes simplex thymidine kinase or cytosine deaminase which, once
20 internalised into the mononuclear phagocytes would act as a reservoir for activation of the prodrugs ganciclovir and 5-fluorocytosine.

Another example of a prodrug activation enzyme is the P450 family e.g. CyP2B6 which activate cyclofosfamide and ifosfamide (30)

Other examples of hypoxia regulatable therapeutic genes are to be found in PCT/GB95/00322 (WO9521927).

Alternatively said hypoxia regulatable agent may comprise a bioreductively activated drug prodrug such as RSU1069 or Tirapazamine which are activated
5 at very low levels of oxygen as well as with contact with enzymes such as reductases. Thus, where not only hypoxia, but protein action, typically enzymic, is required for activation of said regulatable agent, said therapeutic composition of the invention may further comprise an agent that activates said hypoxia regulatable agent, such as a reductase and/or a gene encoding said
10 reductase.

In a further embodiment of the invention said ischaemia or stress regulatable agent may comprise a therapeutic gene, that is a gene encoding a therapeutic agent which is under the control of an ischaemia or stress sensitive agent such as an ischaemia or stress regulated expression element i.e. a promoter or
15 enhancer which is sensitive to any one or more of the factors characterising ischaemia or stress such as, for example, without limitation, glucose deprivation, low pH, elevated levels of lactate or pyruvate and/or ion or osmotic imbalance. Thus, under conditions of ischaemia or stress said elements will be activated so as to enable the gene encoding the therapeutic agent to be
20 expressed. In this embodiment of the invention the binding agent comprises an agent that ensures internalisation of said therapeutic gene with a view to incorporating same into the mononuclear phagocyte genome.

As mentioned above, the ischaemia or stress regulatable agent may comprise a pro-drug activation enzyme as previously described, or a gene encoding same.

Suitable examples of DNA sequences known to be activated by any of the
aforementioned factors, or "stress factors" may be used in the invention and
examples of such sequences include, without limitation, promoter and/or
enhancer sequences activated by glucose deprivation, low pH or elevated levels
5 of lactate or pyruvate and/or ion or osmotic imbalance. For example, the
glucose-regulated proteins (grp's) such as grp78 and grp94 are highly conserved
proteins known to be induced by glucose deprivation (19). The grp78 gene is
expressed at low levels in most normal healthy tissues under the influence of
basal level promoter elements, but has at least two critical "stress-inducible
10 regulatory elements" upstream of the TATA element (19, 20). Attachment to
a truncated, 632-base pair sequence of the 5'end of the grp78 promoter (which
include these two stress-inducible regulatory sacs) confers high inducibility to
glucose deprivation on reporter genes *in vitro* (20). Furthermore, use of this
promoter sequence in a retroviral vector drove high level expression of a
15 reporter gene in tumour cells in murine fibrosarcomas, particularly in central,
relatively ischaemic/necrotic sites (20). In addition it is thought that grp78 may
be inducible by other stress factors (27).

Another group of highly conserved, what may be termed, "stress proteins" are
those responsive to low pH. The genes for these proteins are known to have
20 acid-inducible promoters, examples of which have been cloned and
characterised in recent studies (21) indeed, it was demonstrated that the acid-
inducibility of several such defined DNA sequences in hybrid DNA constructs
consisting of each one of these fused to a reporter gene encoding a green
fluorescent protein was sensitive to pH. Maximum induction was seen when the
25 pH was dropped to 4.5.

Thus, said stress regulatable agent comprises a therapeutic gene under the control of a stress regulated expression element known to those skilled in the art. Notably, a stress regulated expression element is intended to include both homologous and heterologous elements i.e. that is to say promoters and/or
5 enhancers for genes known to be expressed, or over-expressed, by mononuclear phagocytes in stress conditions; and promoters or enhancers for genes known to be over-expressed by other tissues in said conditions, respectively. Examples of the former group include the expression element of the osteopontin gene and thymidine phosphorylase gene. Other examples of homologous
10 promoters or enhancers comprise those involved in the phagocytic activity of macrophages including CD36, CD68, thrombospondin, the $\alpha v \beta 3$ integrin and low density lipoprotein receptors (25, 26).

In a yet alternative embodiment of the invention said agent may be regulated by means other than hypoxia or ischaemia or stress.

15 Indeed, the use of hypoxia or ischaemia or stress to regulate the expression/activity of a therapeutic gene may not be an exclusive feature of the utilisation of mononuclear phagocytes as a means of targeting therapeutic genes to tumours or regions of hypoxia/ischaemia/stress.

In a further embodiment of the invention it may be preferable to place the
20 therapeutic gene under the control of an inducible or repressible promoter element, transcription from which can be modulated to regulate the effective dose of the therapeutic gene. This may result in more accurate control of expression of the therapeutic gene and thereby reduce undesirable side effects

as a consequence of the gene therapy.

By way of example, a constitutively active promoter used to express a therapeutic gene/agent is placed under the regulation of the antibiotic tetracycline by inclusion in the promoter of the tetracycline repressor DNA sequence. The vector DNA incorporating the therapeutic promoter cassette includes the DNA sequence of the tetracycline repressor protein under the control of a suitable promoter element. Transfection of said DNA construct into the mononuclear phagocyte genome results in expression of tetracycline repressor protein and a repression in the transcription of the therapeutic gene.

Transfected mononuclear phagocytes are then re-introduced into a patient to allow said phagocyte to infiltrate, for example, a tumour. Tetracycline is then administered orally, via an intravenous route, or injected directly into the tumour tissue. The administration of tetracycline to the patient results in inactivation of the binding between the tetracycline repressor protein and the operator DNA sequence in the promoter of the therapeutic gene thus enabling its expression. The effective dose of tetracycline can be varied to regulate the expression of the therapeutic gene/agent and thereby optimise the effect of the therapeutic gene/agent.

The system may also be exploited to enable the regulated expression of a "suicide" gene, the expression of which in a mononuclear phagocyte would result in the death of the phagocyte carrying the gene. Selective removal of mononuclear phagocytes may then inhibit revascularisation of tumour tissue thereby restricting tumour development.

This example of regulated expression has been shown to occur in both

prokaryotic (WO 9532295-A) and eukaryotic cells (28, 29) to successfully regulate the expression of both homologous and heterologous DNA sequences. This description is merely to serve as an example of how a prokaryotic sequence can be used to regulate the expression of a eukaryotic gene and is not meant to
5 be exclusively limited to the tetracycline repressor operon.

Alternatively, or in addition, especially where internalisation of said therapeutic composition is required said therapeutic composition may further comprise an internalisation agent so as to ensure that the therapeutic composition is internalised by the mononuclear phagocytes. Agents which are suitable for
10 ensuring internalisation of the therapeutic composition include, but are not limited to, plasminogen activation inhibitors (PAI-1 or PAI-2) or protease nexin (PN).

In yet a further preferred embodiment of the invention said binding agent is adapted to target or bind to any one or more cell surface mononuclear phagocyte molecules such as antigens or receptors. Further, said binding agent may
15 comprise an antibody to any one or more of said molecules such as antigens or receptors, or an effective fragment of said antibody. Alternatively still said binding agent may comprise a suitable ligand either synthetically manufactured or naturally occurring.

20 Alternatively, the binding agent may comprise a vector. The vector may be a non-viral vector. Examples of non-viral vectors include plasmid DNA compacted with a DNA compaction agent such as one containing poly - L-lysine or a liposome or immunoliposome containing plasmid DNA compacted with a DNA - compaction agent (such as a poly-lysine). The non-viral vector

may be a targeted non-viral vector such as plasmid DNA compacted with mannosylated PolyLysine (MPL).

In a yet further preferred embodiment of the invention said binding agent may comprise a viral vector.

- 5 The vector may be a recombinant viral vector such as an adenovirus vector, an adeno - associated viral (AAV) vector, a herpes-virus vector or a retroviral vector in which case gene delivery (transduction) is mediated by viral infection. The viral vector may be a defective viral vector which is not capable of replication in the target cells. The viral vector may also be a chimeric viral
- 10 vector containing components of more than one virus such as retrovirus pseudotyped with the envelope of another virus or an adenovirus capable of expressing retroviral genetic elements (eg. Feng et al 1997 Nature Biotech. 15:866-870). Preferably the viral vector is one which is capable of preferentially transducing non-dividing cells such as a lentiviral vector, including HIV based
- 15 vectors (Naldini et al 1996 Science 272: 263-267). Particularly preferred is a targeted viral vector such as a targeted adenoviral vector capable of preferentially transducing mononuclear phagocytes. Examples of methods for targeting adenoviral vectors to specific cell types are described (eg Krasnykh et al 1996 J. Virol 70: 6839-6846; Wickham et al 1996 J. Virol 70: 6831-6838);
- 20 Stevenson et al 1997 J. Virol. 71: 4782-4790; Wickham et al. 1995 Gene Therapy 2: 750-756. Also preferred is a viral vector which is resistant to human complement, for example by production in a human cell line.

In any event, the viral or non-viral vector will contain a promoter to direct expression of the or each therapeutic gene and may contain additional genetic

elements for the efficient or regulated expression of introduced genes, including enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals. The enhancer may contain elements for regulated expression such as a hypoxia regulated enhancer (for example a
 5 binding element for the transcription factor HIF1) or elements which respond to stress or low glucose. The enhancer elements or elements conferring regulated expression may be present in multiple copies. Combinations of such elements are also envisaged. Genes encoding appropriate transcription factors may also be included in the vector in order to enhance the response to hypoxia,
 10 stress or low glucose. For example a gene encoding HIF1-alpha may be included in the vector (Jiang et al 1996 J Biol Chem 271: 17771-17778).

Additional vector components will be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication, and integration as appropriate using components which are well known in the art.

15 Suitable therapeutic genes may encode proteins which are secreted from the mononuclear phagocyte or which are retained within the cell. For treatment of cancer, suitable therapeutic genes include genes encoding proteins which act as for example: activators of anti-tumour immune mechanisms (such as cytokines, co-stimulatory molecules and immunoglobulins); of angiogenesis;
 20 or which provide enhanced drug sensitivity (such as pro-drug activation enzymes). The use of combinations of such genes is also envisaged. Genes or combinations of genes which show a by-stander effect in the destruction of neighbouring or distant tumour cells are preferred. For treatment of ischaemia, genes encoding proteins which act to stimulate angiogenesis (such as vascular
 25 endothelial growth factor (VEGF)) or other growth factors are suitable

examples.

A brief list of those cell surface molecules that may be targeted by said binding agent is as follows; the receptor for human Urokinase Plasminogen Activator (uPAR; CD87); the receptor for human Colony Stimulating Factor (CSF-1);
 5 CD63; CD64; CD11b; CR3; CD33; the scavenger receptor; all or part of the receptor for the various forms of human monocyte chemoattractant protein (MCP-1, 2, etc); CD14; mannose or mannose-6-phosphate surface receptors; CD16; or HLA-DR.

In a yet further preferred embodiment of the invention said therapeutic
 10 composition comprises any one or more of the drug conjugates illustrated in Table 1, and more preferably, any combination of part I and/or part II and/or part III components specified in Table 1.

According to a yet further aspect of the invention there is provided a delivery system for targeting therapeutic compositions to hypoxic and/or ischaemic
 15 and/or stress sites comprising a hypoxia and/or ischaemia and/or stress regulatable agent and an agent for controlling the functional effectiveness thereof, and coupled thereto, a binding agent for a cell surface molecule of a mononuclear phagocyte.

According to a yet further aspect of the invention there is provided a method for
 20 targeting desired agents to hypoxic and/or ischaemic and/or stress sites comprising;

- (i) coupling at least one of said agents to a binding agent that is adapted for

- binding or targeting a cell surface molecule expressed by a mononuclear phagocyte;

- (ii) exposing said coupled agent to mononuclear phagocytes; and
 - (iii) allowing said mononuclear phagocytes to migrate, under conditions that
- 5 support migration, either *in vitro* or *in vivo*.

According to a yet further aspect of the invention there is provided a method for treating conditions associated with hypoxic and/or ischaemic and/or stress states comprising administering to an individual to be treated the therapeutic composition of the invention.

- 10 According to a yet further aspect of the invention there is provided a method for treating conditions associated with hypoxic and/or ischaemic and/or stress states comprising withdrawing blood and/or serum from an individual to be treated and treating said blood and/or serum *in vitro* with a hypoxically and/or ischaemically and/or stress inducible therapeutic gene under conditions that
- 15 enable incorporation of said gene into the nucleus of mononuclear phagocytes and re-injecting said treated blood and/or serum into the individual either systemically or directly into a hypoxic and/or ischaemic and/or stress area.

- 20 According to a yet further aspect of the invention there is provided mononuclear phagocytes which have coupled thereto, or internalised therein, at least a hypoxia and/or ischaemia and/or stress regulatable agent and, optionally, an agent that is adapted to bind to a mononuclear phagocyte ligand which is typically found on the cell surface of said mononuclear phagocyte.

According to a yet further aspect of the invention there is provided a method for selectively destroying a mononuclear phagocyte comprising attaching thereto or internalizing therein a cytotoxic, hypoxically and/or ischaemically and/or stress activated agent and exposing said mononuclear phagocyte to hypoxic
 5 and/or ischaemic and/or stress conditions that occur either artificially by induction or occur/exist naturally.

In the instance where hypoxia and/or ischaemic and/or stress occurs/exists naturally said mononuclear phagocyte migrates in a normal manner to said hypoxic and/or ischaemic and/or stress area so that the said agent is only
 10 activated at a target area. In this way the potentially deleterious effects of mononuclear phagocytes in tumours is obviated. Moreover, having regard to the nature of said agent a bystander effect may be achieved, for example where said cytotoxic agent is released on death of said mononuclear phagocyte it may have a further deleterious effect on the hypoxic and/or ischaemic and/or stress
 15 tissue, such as, but not limited to, tumour tissue.

Many of the preferred embodiments hereinbefore described represent appropriate modifications of any one or more of the above referred to further aspects of the invention.

Embodiments of the invention will now be described by way of example only
 20 with reference to the following Figures and Table wherein:

FIGURE 1 shows the distribution of macrophages in areas of high and low vascularity in 46 invasive breast carcinomas (i.e. assessed by quantification of

CD31 postivity of blood vessels; see reference 3).

FIGURE 2 shows association of macrophage index with necrosis in 92 breast carcinomas. * w.r.t Grade O group (Mann Whitney U Test). Number of data points in each group is indicated at the base of each column. [Grades for necrosis: 0, no necrosis; 1, few focal areas of necrosis; 2, many focal areas of necrosis; 3, almost entirely necrotic].

FIGURE 3 shows the effect of clamp - induced hypoxia (induced by tumour vessel clamping for 2h) on macrophage infiltration in human breast cancer xenografts grown in nude mice;

FIGURE 4 shows the diagram of an Adenovirus transfer vector (QBI-HRE-LacZ) containing a LacZ gene under the control of a hypoxia regulated promoter – enhancer;

FIGURE 5. Macrophage infiltration into hypoxic areas in tumour spheroids (i.e. an in vitro model of tumour hypoxia).

- (i) shows the oxygen profile across a tumour cell spheroid. All but the cells in the outer 100 μm of these 3-D cultures are hypoxic (i.e. experiencing oxygen levels of 0-15 pO_2 mmHg; a level equivalent to that present in hypoxic/necrotic sites in human tumours). This hypoxia is produced by the inability of oxygen to diffuse into the central areas of spheroid. The glucose profile of the spheroid is similar to that seen for oxygen.
- (ii) shows two different tumour spheroids (made of the breast cancer cell line, MCF-7) following co-culture for 24h with the

monocytic cell line, U937. The U937 cells (darkly stained cells labelled with a monoclonal antibody to the pan-macrophage marker, CD68) accumulate in the hypoxic rim of viable, but hypoxic tumour cells around the central areas of necrosis ("N").

- 5 (iii) shows the infiltration into tumour spheroids of U937 cells preloaded with fluorescent dye. The top panel is a light micrograph showing the opaque central area of necrosis ("N") which forms in these spheroids as a consequence of nutrient (e.g. oxygen, glucose etc) deprivation. The bottom panel is the same
- 10 spheroid a fluorescent microscope to show the presence within the spheroid of the fluorescent (i.e. light coloured cells) U937 cells. The latter take up a similar position to that seen in (ii), i.e. they congregate in a collar of hypoxic tumour cells around the central areas of necrosis.

15 FIGURE 6 illustrates cloning of HRE's in a receptor plasmid.

Table 1 represents specific examples of drug conjugates.

Materials and Method

A preferred way of working the invention will now be described with particular reference to the use of mononuclear phagocytes to target therapeutic genes to

20 hypoxic sites in human tumours. However, it will be understood, that this illustration of the invention ie by way of using mononuclear phagocytes to target therapeutic gene delivery is just one way in which the invention may be worked. Moreover, this example of the invention ie the targeting of therapeutic

agents to human tumours is, yet again, just one example of how the invention may be worked.

Example 1 - Gene transfer into monocytic cell lines

Retroviral transfer into the monocytic cell line U937.

- 5 Techniques for retroviral vector mediated gene transfer into U937 cells (obtained from ATCC) were optimised with the marker gene β -galactosidase using retroviral particles packaged with two different envelopes. Retroviral constructs were packaged in a transient three plasmid system (Soneoka et al 1995 NAR 23 628-633); pHIT111 CMV lac Z genome with the 4070A amphotropic envelope
- 10 (Soneoka et al) and the MFG CMV nuclear localisation sequence lac Z genome pseudotyped with VSV-G (Cosset et al J Virol 1995 69 7430-7436). Retroviral vectors were transduced into U937 at multiplicity of infection of 1-3 in the presence of 8 μ g/ml polybrene in maintenance media (RPMI/10% FCS, obtained from Sigma) for 2h at 37°C. Cells were stained using standard X-gal
- 15 histochemical techniques 48h later (MacGregor et al 1991 p217-235, Methods in Molecular Biology, Vol 7 Ed E J Murray). Levels of transduction were approximately 0.5 % with the 4070A amphotropic envelope and approximately 1% pseudotyped with VSV-G. Transduction approaches may be increased using repeat transduction with high titre viral preparations concentrated using
- 20 standard cross-flow filtration methods (following manufacturers protocol supplied by Flowgen) and co-culture with producer cell lines (Morgenstern and Land 181-206, Methods in Molecular Biology, Vol 7 Ed E J Murray). Cells transduced with pHIT111 were selected in 1 mg/ml G418 (Gibco) resulting in the isolation of stable β -galactosidase positive cell lines as determined by X gal

staining. FACS based selection approaches have been established using the fluoresceinated β -galactosidase substrate FDG (fluorescein di-b-D-galactoside) according to the manufacturers protocol (Molecular Probes).

Construction of hypoxically regulated retroviral genome.

- 5 To produce a hypoxically regulatable retroviral construct long a terminal repeat (LTR) plasmid was generated from the retroviral plasmid pLNSX (Miller and Rosman 1989 Biotechniques 7 980-990) by cutting at the Nhe1 sites within the LTR, removing the majority of the intervening retroviral genome sequences and religating the backbone. This produces an LTR plasmid in which enhancer and
- 10 enhancer/promoter swaps can be engineered. The retroviral enhancer was exchanged with the PGK hypoxia response element (HRE) by performing an Nhe1/Xba1 swap. The resultant vector was then recut with Nhe1 and the Nhe 1 fragment of the retroviral genome designated MOI was inserted. MOI was generated from the MFG vector (Bandara et al 1993 PNAS 90 10764-10768) by
- 15 the generation of a minimal functional packaging signal using PCR with the following primers:

HindIIIIR: GCATTAAAGCTTTTGCTCT

L523: GCCTCGAGCAAAAATTCAGACGGA

- The fragment generated contains MMLV nucleotides +1-+523 and thus does not
- 20 contain gag coding sequences that start at +621 (numbering according to Shinnick et al 1981 Nature 293 543-548). This fragment can be isolated as a HindIII/Xho1 fragment and used to replace the corresponding region of MFG thus removing gag coding sequences. The vector also includes the SD and SA sites spanning the intron of MFG and and FMDV internal ribosome entry site
- 25 (IRES) sequence to allow coincidental translation of two coding sequences from

a single bicistronic mRNA. A nuclear localising lac Z (including the nls sequences from SV40 large T antigen; Bonneret 1987 PNAS 84 6795-6799) was cloned immediately 3' to this in the Stu1/Xho1 sites.

Electroporation of monocytic cell lines

- 5 Electroporation conditions were optimised on the U937 and THP-1 (obtained from ATCC) monocytic cell lines using β -galactosidase and luciferase reporter constructs based on the pGL3 vector series (Promega).
Optimal conditions using the Bio Rad Gene Pulser II were determined as follows;
- 10 1×10^7 cells were washed and resuspended in 500 μ l serum free RPMI 1640 (Sigma) at room temperature then transferred to a 0.4cm electroporation cuvette. 30 μ g DNA was added and the cuvette pulsed at 300V, 950 μ F giving time constants of between 20-25 milliseconds. Cells were rested at room temperature for 10 mins then seeded into 2ml of RPMI/10% FCS (Sigma) in a 6-well plate
- 15 and incubated at 37°C, 5%CO₂.
Transfection efficiencies were assayed by standard X-gal histochemistry (MacGregor et al 1991 p217-235, Methods in Molecular Biology, Vol 7 Ed E J Murray) and Dual reporter luciferase assay (according to manufacturers protocol obtained from Promega). Efficiencies were typically 2-5% for THP-1
- 20 and 8-14% for U937. Stable transfectants were selected either by culture in 1mg/ml G418 (Gibco) or using FDG FACS sorting (see above).

Example 2 - Gene transfer to primary human macrophages.

Primary human macrophages were isolated from leukocyte enriched blood

(obtained from the Blood Transfusion Service, Southmead Rd, Bristol) as follows; peripheral blood mononuclear cells were obtained by centrifugation through a Ficoll gradient (Pharmacia) according to the manufacturer's instructions. Macrophages were obtained from this cell population by
 5 adherence on tissue culture plastic over 7 days in RPMI 1640 (Dutch modified, Sigma) + 2% heat inactivated human AB serum (Sigma) or 10% FCS. In some experiments, isolated macrophages were subsequently cultured with the addition of 100U/ml GM-CSF (obtained from R and D systems).

Recombinant adenovirus mediated gene transfer to primary human

10 Macrophages.

Recombinant adenoviruses were created using the Quantum Biotechnologies Adenoquest™ Kit (NBL Gene Sciences) according to the manufacturers instructions. Briefly, the cDNA/reporter construct of interest (e.g hypoxia response element nls LacZ reporter cassette) was cloned into the pADBN
 15 transfer vector (Figure 4) The vector was linearised near the Inverted Terminal Repeat (ITR) to ensure efficient replication of the recombinant virus and was co-transfected along with the long arm of the adenoviral vector (QBI-viral DNA) into human 293 cells. Recombination in 293 cells between the homologous regions of linearised transfer vector and adenovirus genome vector
 20 produced complete adenoviral recombinants. The recombinants formed plaques in the 293 monolayer which were picked as agarose plugs, purified by plaque assay and Arkclone P centrifugation and characterised by PCR (Protocols for gene transfer in neuroscience:

Towards gene therapy of neurological disorders, Chapter 8, Ed. Lowenstein &
 25 Enquist (1996) Wiley & Sons). An example of such a recombinant adenovirus

is QBI-HRE-LacZ shown in Figure 4.

Recombinant adenovirus, QBI-AdenoLacZ, containing the CMV LacZ reporter construct (4×10^8 PFU/ml, Quantum Biotechnologies Adenoquest™ Kit, NBL Gene Sciences) was used to infect primary macrophages seeded at a density of
 5 7×10^5 / well in a 12 well plate. A multiplicity of infection of 80 gave an infection efficiency of 10-20% after incubation for 24 hours in the standard macrophage culture medium.

Gene Transfer to cultured primary macrophages using mannosylated PolyLysine.

10 Differentiated macrophages express several cell-surface lectins/receptors including the mannose-6-phosphate receptor (Shepherd et al. 1982 J. Reticuloendothel. Soc 32: 423-431). Primary macrophages cultured for 7-14 days in the presence of GM-CSF (see above) were transfected using the pGL3 control plasmid (obtained from Promega with the luciferase coding sequence
 15 replaced by that for β -galactosidase) using mannosylated poly-L lysine:DNA complexes according to published methods (P. Erbacher et al, Human Gene Therapy 7: 721-729). This yielded 20% transfectants as determined by X-gal histochemistry (MacGregor et al 1991 p217-235, Methods in Molecular Biology, Vol 7 Ed E J Murray).

20 Construction of plasmids containing HREs

Sequences from a region approx. 300-375 bp upstream of the transcription start of the human Enolase A gene were chosen containing three HIF-1 consensus binding sites (Semenza et al 1996 J. Biol. Chem. 271: 32529-32537. The

following oligonucleotides were synthesised to assemble this sequence and add a BglII site at the 5' end and a BamHI site at the 3' end.

Lead strand (75-mer)

GATCTGAGGGCCGGACGTGGGGCCCCAGAGCGACGCTGAGTGCGTG
 5 CGGGACTCGGAGTACGTGACGGAGCC
 CCG

Complementary strand (75-mer)

GATCCGGGGCTCCGTCACGTACTCCGAGTCCCGCACGCACTCAGCGT
 CGCTCTGGGGCCCCACGTCCGGCCC
 10 TCA

The oligonucleotides were inserted into the pGL3-pro vector (Promega) at the BglII site (see Figure 7). Insertion of these oligonucleotides at this site leaves a unique BamHI site downstream of the luciferase gene for insertion of additional transcription units. For example, a transcription unit encoding HIF-1
 15 alpha can be inserted at this site in order to further enhance the response to hypoxia.

Similarly, oligonucleotides were synthesised representing the HRE from the murine LDH gene (Firth et al 1995 J. Biol. Chem. 270: 21021-21027. The sequence chosen lies 15bp upstream of the LDH TATAA box. It contains a
 20 HIF-1 consensus binding site and a putative cyclic AMP-response element. This HRE was also introduced at the BglII site of pGL3-pro.

Lead strand (56-mer)

GATCTCTACACGTGGGTTCCTCGCACGTCCGCTGGGCTCCCACTCTGA
 CGTCAGCGG
 25 Complementary strand (56-mer)

GATCCCGCTGACGTCAGAGTGGGAGCCCAGCGGACGTGCGGGAACC
CACGTGTAGA

Cloning of the human grp78 promoter

The promoter was cloned by PCR amplification from human genomic DNA and
5 includes the complete promoter / enhancer sequence and 5' UTR of the GRP78
gene Chuck et al (1992). Nucleic Acids Research. 20: 6481-64; EMBL Data
Library accession no. for the GRP78 promoter sequence: X59969). The
amplified fragment corresponds to bases 6-585 of this sequence. The primers
used in the amplification reaction incorporated an AseI site at the 5' end and a
10 XhoI site at the 3' end. The grp78 promoter fragment was then cloned into
these sites present in a Clontech pEGFP-N1 vector allowing expression of beta-
galactosidase / GFP fusion protein.

Example 3 - Infiltration of multi-cellular human tumour spheroids with human macrophages (U937 cells or monocytes).

15 Tumour spheroids were established in culture using the MCF-7 cell line
(ATCC) using the following procedure.

A. Establishment of Spheroid Cultures

1. Uniformly sized spheroids were grown in standard 96-well tissue
culture plates.
- 20 2. A 1.5% solution of agarose was prepared in media and autoclaved (the
media should not contain any supplements or foetal calf serum as this
causes the formation of bubbles and the cells will plate down and not
form spheroids).

3. 100µl of the agarose was aliquoted into each well and allowed to cool.

Plates were then warmed to 37°C before use.

4. Monolayers of tumour cell lines were stripped in the exponential growth phase, resuspended and counted using a haemocytometer. The cells were then diluted to the appropriate number of cells for spheroid initiation. For T47D and HT29 this was 1000 cells per well and for MCF-7 it was 2000 cells per well. Each well was filled with 200µl of the cell suspension. The final concentration of the cell suspension for T47D was 5000 cells per ml. (NB spheroids were grown in the media used normally for each of the cell lines (eg T47D are grown in DMEM supplemented with antibiotics and fungicides).
5. Following initiation, the spheroids were incubated at 37°C in a CO₂ incubator and left undisturbed for 5 days to allow aggregation to occur.
6. Spheroids were fed fresh medium three times per week.

15 B. Co-culture of spheroids with macrophages

1. Monocytic cell lines (eg U937 cells), peripheral blood monocytes or monocyte-derived macrophages were introduced into the spheroid culture once the spheroids have formed necrotic centres. This stage depends upon the cell line used. For MCF-7 and T47D it was after 2-3 weeks of culture, when a dark area can be seen in the centre of the spheroid.
2. This was done by removing 100 µl of media from the wells and replacing it with a suspension of macrophages (50,000 per well). For cell lines, the change in media was not a problem but for PMBC the media should be serially changed for both the spheroid and the macrophage until they are in the same media. These cells infiltrated the spheroids in the first hour

of co-culture and continued to do so for up to 48 hrs. After this, the spheroids were removed from the wells using a glass Pasteur pipette, placed in a test tube and rinsed in PBS to remove any loose macrophages or cell debris.

- 5 3. The spheroids were then allowed to settle to the bottom of the tube, the PBS removed and the spheroids processed for paraffin embedding (or frozen in OCT).

The results of an experiment showing infiltration of MCF-7 spheroids with U937 cells is shown in Figure 3. Each spheroid displays the typical central area of necrosis ('N') surrounded by a collar of hypoxic tumour cells (approx 5 - 10 cells in thickness) and then several outer layers of cells that are relatively normoxic. U937 cells, immuno-labelled (dark staining) for the pan macrophage marker CD68, can be seen accumulating in the hypoxic tumour cell layers around the central necrosis.

15 **Example 4 - demonstration of infiltration into spheroids of monocytic cells labelled with a fluorescent dye.**

U937 cells were incubated with 90µl of 4-(4-(didecylamino)styryl)-N-methyl pyridium iodide (2mg/ml in abs ethanol) for 45 minutes then washed to remove the excess dye. Multicell spheroids (5-600µm in diameter) were placed in a bacterial petri dish (agarose would fluoresce and they would adhere to tissue culture plastic) and incubated with 75000 dye-loaded U937 cells per spheroid. The final volume was 15 mls. After 4 days of co-culture, spheroids were washed to remove unattached macrophages and photographed using a fluorescence microscope.

The result is shown in Figure 6. Panel A in Figure 6 shows a section through a spheroid photographed under white light. The central necrotic core (N) is visible as a dark area. Panel B, photographed under fluorescence optics, shows accumulation of macrophages labelled with fluorescent dye, in the hypoxic region around the necrotic core.

C. Paraffin embedding of spheroids

1. Spheroids were immersed in formalin for 2 hrs or overnight.
2. They were then either embedded in agar and processed or placed in a piece of pre-folded tissue paper which was then folded and placed in a tissue-processing cassette. Alternatively a Cellsafe biopsy cassette was used (a mesh chamber in which the spheroids are placed). The cassette was then closed and placed inside the processing cassette. The mesh prevented the spheroids escaping.
3. The spheroid preparations were processed through ascending grades of alcohol to paraffin wax using a Citadel 2000 Histopathology Processing Unit.
4. Sections of the paraffin wax blocks were cut using a microtome onto coated slides.

D. Immunohistochemistry for CD68 (a pan macrophage marker) to localise macrophages in spheroids

1. Spheroid sections were dewaxed in xylene and absolute alcohol.

2. The endogenous peroxidase in the sections was then blocked with 2% H₂O₂ in methanol for 10 mins.
3. Antigen retrieval: sections were exposed to proteinases type XXIV for 15 mins at 37°C.
- 5 The sections were then incubated in the following: (with 3x5 min washes between each step)
 4. Normal serum for 30 mins at room temperature
 5. Primary anti-CD68 monoclonal antisera at a dilution of 1:100 for 1hr at room temperature or overnight at 4°C.
 - 10 6. Secondary antibody (biotinylated horse anti-mouse IgG) for 30 mins at room temperature followed by an avidin-biotin-peroxidase complex for 30 minutes at room temperature (ie using the Vector ABC Elite kit)
 7. Visualized with the chromagen, DAB or AEC for 20 10 mins.
 8. The nuclei were then counterstained with haematoxylin and sections
 - 15 mounted with coverslips for viewing.

NB Spheroid sections needed to be washed thoroughly in diluent between each step of the staining protocol to limit background / non-specific staining.

Example 5 - Effect of clamp - induced hypoxia on macrophage infiltration into tumour xenografts.

- 20 In order to determine the effect of hypoxia on macrophage infiltration into tumours in vivo, a xenograft model was established in which a human breast-tumour derived cell line is grown under the skin in nude mice. Clamping of the

tumours induces increased hypoxia in the tumours.

A. Implantation and clamping of breast tumour xenografts in nude mice

(as described in Griffiths L et al 1997 Cancer Res 57: 570 - 72)

- 5 1. Male nu/nu mice were injected s.c. with 5×10^7 MDA-231 cells in the dorsal area in a volume of 0.1ml. Twelve animals were used per experimental group.
- 10 2. When the tumours reached 600mm³ (ie after 6 - 7 weeks of growth) they were clamped for 2h to occlude the blood supply and induce radiobiological hypoxia (<1% oxygen). Control tumours were not clamped. (NB this procedure only partially occludes the blood supply to the tumour, thereby allowing continued macrophage infiltration into the tumour).
- 15 3. Tumours were excised immediately following clamp removal, fixed and paraffin-embedded for routine histology and/or immunohistochemistry for the pan murine macrophage marker, F4/80.

B. Immunohistochemistry for the pan murine macrophage marker.

F4/80

- 20 1. Sections were dewaxed and rehydrated through graded alcohols to distilled water.
2. Antigen retrieval: Serotec's reagent, STUF, was used to unmask antigen (as described by the manufacturers).
3. Slides were then washed in PBS/0.1% Triton-X-100: 3 changes x 5 mins each.

4. Endogenous peroxidase was blocked in sections in 2% H₂O₂ / methanol for 20 mins.
5. Sections were then washed as in Step 3.
6. Non specific protein binding sites in sections were blocked using 5% normal rabbit serum in PBS for 30 mins.
7. Sections were then incubed in Serotec's F4/80 diluted to 1:20 in normal rabbit serum (5% NRS in PBS) for 90 mins at RT.
8. Sections were then washed as in Step 3.
9. The Vector biotinylated rabbit anti-rat (mouse absorbed) at 1:100 in PBS was then placed on the sections for 45 mins at RT.
10. Sections were then washed as in Step 3.
11. Sections were then incubated in Vector ABC reagent for 45 mins at RT.
12. Sections were then washed in Step 3.
13. The brown colour reaction was then developed with Vector DAB kit for 2 mins.
14. The sections were then washed in water and nuclei was counterstained in haematoxylin.
15. After dehydrating through alcohols, cleared in Xylene and mounted with coverslips, the number of F4/80 positive macrophages was then estimated for each tumour by taking the average of the counts from 20, randomly selected fields of view (x20 objective) obtained using a SeeScan image analysing device.

The results of this experiment are shown in Figure 3. Clamping of the tumours led to increased infiltration of macrophages, demonstrating a correlation between the degree of hypoxia and the number of infiltrating macrophages.

Further Examples of Therapeutic Compositions For use in Working the Invention

Table 1 represents examples of drug conjugates for attaching to, or internalising in, mononuclear phagocytes.

- 5 The drug conjugate of choice can be infused (repeatedly or as a single injection) into the general circulation so as to bind *in vivo* to the surface of systemic mononuclear phagocytes and/or macrophages already resident in diseased tissues (e.g. malignant tumours). In support of the aforementioned mode of drug delivery is the finding that when a monoclonal antibody, specific for the
- 10 Mac-1 antigen on macrophages, is conjugated to indium (a radioactive element) and injected intravenously into mice bearing a solid tumour, it was seen on scintographs to accumulate predominantly in the cancer lesion (9). Alternatively the drug conjugate can be exposed to monocytes *ex vivo*, following their purification from the blood of patients using such standard
- 15 methods as Ficoll- Hypaque gradients and elutriation as described previously in (10).

- Homing of blood monocytes loaded up with drug conjugates into malignant tumours can be augmented by prior treatment with conventional systemic therapies which induce local inflammation/necrosis in the diseased tissue (e.g.
- 20 radiotherapy or chemotherapy in the case of cancer patients). This stimulates the release of chemoattractant factors for monocytes/macrophages such as MCP-1 (11, 12) and would thus enhance the delivery and hence the therapeutic efficacy of the drug conjugate at the diseased site.

Mode of production of selected drug conjugates

Drug 1. (with reference to Table 1) *RSU1069 - F(ab)₂ of a monoclonal antibody to CD87 (uPAR)*

This conjugate uses a highly specific F(ab)₂ fragment a monoclonal antibody to
5 CD87 (urokinase plasminogen activator receptor; uPAR) to target naturally occurring uPAR on the surface of monocytes and macrophages.

A monoclonal antibody to CD87 is made as described in (13) and then
cleaved/purified to a specific F(ab)₂ monoclonal antibody fragment using
standard proteolytic methods. Depending upon the part of uPAR used to raise
10 the antibody (i.e. as the antigen), the epitope for the antibody generated may either be in the (i) ligand (i.e. uPA) -binding portion of the uPAR (in which case the drug conjugate will only bind to unoccupied uPAR on monocytes/macrophages), or (ii) the non ligand (i.e. uPA) -binding portion of the uPAR (in which case the drug conjugate will bind to both unoccupied and
15 unoccupied uPAR on monocytes/macrophages). The most effective drug uptake is likely to be achieved using the latter form of conjugate.

The fragment of the CD87 monoclonal antibody is conjugated to the
bioeducative prodrug, RSU1069, by the latter being reacted with an agent such
as arylazide to add a N-hydroxysuccinimide group. This is then cross-linked at
20 neutral pH to the CD87 antibody fragment via amine groups to form a conjugate. This method is well established for conjugating drugs to proteins and is described fully in (14).

Drug 2. (with reference to Table 1) *i.e.* RSU1069 - PAI-2

This conjugate uses the affinity of plasminogen activator inhibitor 2 (PAI-2) for urokinase plasminogen activator receptor (uPAR) - urokinase plasminogen activator complexes to target the bio-reductive prodrug to the surface of monocytes and macrophages. PAI-2 triggers the internalization of uPAR-uPA complexes, so the internalization by these cells of the bio-reductive prodrug attached to PAI-2 is assured.

Naturally occurring PAI-2 is obtained from the culture supernatant of human blood monocytes stimulated maximally with interleukin 1 or 2 as described in (15). This is then purified to homogeneity in the usual manner by elution from an anti-PAI-2 immunoaffinity column. Alternatively, PAI-2 can be produced in a recombinant expression system and purified according to the method of (16). The PAI-2 preparation is then conjugated to RSU1069 using the method outlined as hereinbefore described for drug 1(14).

Drug 6. (with reference to Table 1) *i.e.* Interleukin-2 (IL-2) gene linked to hypoxia responsive promoter.

This gene is transferred to monocytes/macrophages using a replication-defective adenoviral vector. Efficient transfer of genes into human macrophages has been achieved with this method with expression of the gene in 40-80% of the cells exposed to the vector and lasting up to 3 weeks after gene transfer (10).

Defective retroviral vectors, direct DNA internalization or such non-gene viral gene transfer systems can also be used such as cationic lipids, liposomes, lectins

or polymers. Genes other than IL-2 which could be of therapeutic benefit include such other immunomodulators as TNF α , or interferon gamma, prodrug activating enzymes, enzyme inhibitors, tumour antigens (to provoke the hosts immune reactivity to the tumour) and anti-oncogenes (antibodies or antisense RNA). It will be understood by those skilled in the art that the DNA construct used may, embody a number of these genes rather than just one and is not intended to limit the scope of the application.

The hypoxically inducible expression control sequence (promoter) for the Epo or PGK genes (or multiple copies thereof) is/are coupled to one or more of the gene sequences of choice (e.g. IL-2 gene sequence) as described by one of us previously in (17).

Ex vivo gene transfer:

The method outlined in (10) involves incubating (under sterile conditions) freshly isolated blood monocytes or monocyte-derived macrophages (monocytes incubated overnight in teflon bags or on plastic cultured wells) in the presence of 100 plaque-forming units per cell of the purified replication-defective vector, Ad.RSV Δ , harbouring the hypoxia-responsive promoter-IL-2 gene construct in RPMI incubation medium (1ml/10⁶ cells). Gene transfer may be enhanced by simultaneous treatment of the cells with 100U/ml of human interferon gamma. Cells are then washed to remove free viral particles and reinterferon gamma and reincubated at 37°C in fresh RPMI medium in teflon bags. The same adenoviral vector but harbouring the E.coli β -galactosidase gene (Ad.RSV. β gal) instead of the hypoxia-responsive promoter-IL-2 gene construct is used as a reporter gene (i.e. to check the efficiency of this gene

transfer method to monocytes/macrophages *ex vivo*). The presence of the β -gal enzyme in cells after infection with Ad.RSV. β gal is then assessed using histochemical methods as described in (17). The transfected cells (10^8 to 10^9 cells) are then injected sterile back into the bloodstream or directly into the
 5 appropriate diseased tissue (e.g. malignant tumour) of the donor as in (10).

In vivo gene transfer:

This is performed according to the *in vivo* method of adenoviral gene delivery described in (18). This involves injecting the vector (i.e. Ad.RSV β) bearing the hypoxia-responsive promoter-IL-2 gene construct into the bloodstream
 10 (primarily to label monocytes) and/or into the diseased tissue (at 10^9 to 10^7 plaque-forming units) to label tissue macrophages.

The invention is exemplified by the drug conjugate candidates in Table 1. It will be understood by those skilled in the art that such conjugates represent selected examples and are not intended to limit the scope of the invention,
 15 furthermore it will be understood that indeed any one example of a candidate Part I drug conjugate may, where appropriate, be used in conjunction with any one example of a Part II candidate and/or any example of a Part III candidate. Additionally it will be understood that any example of a Part III candidate may be used in conjunction with any example of a Part II and/or Part I candidate
 20 drug conjugate.

The invention hereinbefore described therefore represents a most elegant and effective means and method of delivering a therapeutic and/or cytotoxic agent to a hypoxic or ischaemic or stress site by use of monocytes and/or

macrophages and their natural ability to congregate at a hypoxic site.

TABLE 1 - SPECIFIC EXAMPLES OF DRUG CONJUGATES

	<u>Part I</u>	<u>Part II</u>	<u>Part III</u>
<u>Drug 1</u>	<i>RSU1069</i>	<i>F(ab)2 to CD87 (uPAR)</i>	-
5	or Tirapazamine		
<u>Drug 2</u>	<i>RSU1069</i>	<i>PAI-2</i>	-
10	or Tirapazamine	(binds to receptor bound urokinase plasminogen activator (uPAR) & ensures internalization of drug)	
<u>Drug 3</u>	<i>RSU1069</i>	<i>PAI-2</i>	<i>NADPH: P450</i>
15	or Tirapazamine	(binds to receptor bound uPAR & ensures internalization of drug)	<i>reductase</i> (reductase for activation of <i>RSU1069</i> or Tirapazamine under hypoxia)

	<u>Drug 4</u>	<i>RSU1069</i> or <i>Tirapazamine</i> on surface of monocyte/ macrophages)	<i>F(ab)2 to CSF-1 receptor</i> <i>(binds to human CSF receptor</i> <i>reductase</i>
5	<u>Drug 5</u>	<i>Cytosine</i> <i>deaminase</i> (prodrug activating enzyme)	<i>F(ab)2 to CD63</i> -
10	<u>Drug 6</u>	<i>Gene for Inter-</i> <i>leukin 2</i> (immuno- <u>stimulatory</u> <u>cytokine</u>) in <i>Ad.RSV</i>	<i>Hypoxia.</i> <i>or ischaemia</i> <i>or stress</i> <i>-resp. promoter</i> <i>sequence</i>
15			

5	<u>Drug 7</u>	<i>DNA sequence for soluble domain of VEGF receptor (eg. flk-1) in Ad. RSV</i>	-	<i>Hypoxia. or ischaemia or stress -resp. promoter sequence</i>
10	<u>Drug 8</u>	<i>A therapeutic gene for example as described in PCT/GB95/00322 WO9521927</i>	<i>pGL3-pro plasmid and mannosylated poly-L lysine</i>	<i>Hypoxia or ischaemia or stress -resp. promoter sequence</i>
15	<u>Drug 9</u>	<i>A therapeutic gene for example as described in PCT/GB95/00322 WO9521927</i>	<i>Adenoviral vector for example QBI-HRE-LacZ</i>	<i>Hypoxia or ischaemia or stress -resp. promoter sequence</i>

REFERENCES

1. O'Sullivan C and Lewis CE (1994) *J. Pathol.* 172: 229-235.
2. Leek RD, Lewis CE, Whitehouse R and Harris AL. (1996) *Cancer Research* 56:4625-4629.
- 5 3. Knighton DR, et al. (1983) *Science*. 221: 1238-85
4. Vaickus L and Foon KA. (1991). *Cancer Invest.* 9:195-209.
5. Byers VS and Baldwin RW. (1988). *Immunol.* 65: 329-409
6. Sands H. (1988). *Antibody Immunoconjug. Radiopharm* 1:213-226.31.
7. Epenetos AA. et al. (1986). *Cancer Res.* 46:3183-91.
- 10 8. Derbyshire EJ and Thorpe PE. (1996). In "*Tumour Angiogenesis*" Eds R. Bicknell, CE Lewis & N. Ferrara. Oxford University Press, Oxford. (in press).
9. Collette B. et al. (1988). *Cancer Immunol. Immuno. Ther.* 26: 237-242.
10. Haddada H., et al. (1993). *Biochem. Biophys. Res. Commun.* 195: 1174-83.
- 15 11. Mantovani A., et al. (1993). *Res. Immunol.* 144: 280-83.
12. Martinet N., et al. (1992). *Cancer.* 70: 854-60.
13. Ronne E., et al. (1991). *FEBS Letts.* 288: 233-36.
14. Gabor F., et al. (1995). *Archiv. der Pharmazie.* 328: 775-80.
- 20 15. Gyetko M. R., et al. (1993). *J. Leuk. Biol.* 53: 598-601.
16. Steven F., et al. (1991). *Eur. J. Biochem.* 196: 431-8.
17. Ratcliffe P. J., Firth J. D., and Harris A. L. (1994). Patent application entitled "Targeting gene therapy". PCT/GB95/00322.
18. Magovern C. J., et al. *Ann. Thorac. Surg.* 62: 425-33.
- 25 19. Attenello J. W., Lee A.S., (1984). *Science* 226:187-1909.
20. Gazit G., Kane S. E., Nichols P., Lee A.S., (1995). *Cancer Res.* 55:

1660-63.

21. Valdivia R. H., Falkow S., (1996). *Molecular Microbiology* 22: 367-78.
22. Brown L.F., Papadopoulossergiou A., Berse B., Manseau E. J., Tognazzi K., Perruzzi C. A., Dvorak H. F., Senger D. R., (1994). *AM. J. Pathol.* 5 145: 610-623.
23. Hijiya N., Setoguchi M., Matsura K., Higuchi Y., Akizuki S., Yamamoto S., (1994). *Biochem. J.* 303: 255-262.
24. Takahashi Y., Bucana C. D., Liu W. B., Yoneda J., Kitadi Y., Cleary K. R., Ellis L. M., (1996). *J. Natl. Canc. Inst.* 88: 1146-1151.
- 10 25. Griffiths L., Dachs GU., Bicknell R., Harris A. L., & Stratford I., (1997). *Cancer Res.* 57 (in press; Feb. issue).
26. Savill J. (1996). *Biochem. Soc. Trans.* 24: 1065-69.
27. Sugawara S., Takeda K., Lee A., Dennert G., (1993). *Cancer Res.* 53: 6001-5005.
- 15 28. Wirtz E and Clayton C. (1995). *Science* 268: 1179-1183.
29. Schmid S. L. (1995). *Trends in Cell Biology* 5: 267-268.
30. Chen L. et al (1996) *Cancer Research* 56:1331-1340.
31. Negus R. P., Stamp G. W., Hadley J., Balkwill K., (1997) *Am. J. Pathol.* 150:1723-1734.

CLAIMS

1. A therapeutic composition comprising a regulatable agent and an agent that binds to a cell surface element of a mononuclear phagocyte.
2. A therapeutic composition according to Claim 1 wherein said regulatable
5 agent is a hypoxia and/or ischaemia and/or stress regulatable agent.
3. A therapeutic composition according to any preceding Claim wherein said regulatable agent comprises a therapeutic gene.
4. A therapeutic composition according to Claim 3 wherein said gene is under the control of a hypoxia and/or ischaemia and/or stress sensitive
10 agent.
5. A therapeutic composition according to any preceding Claim wherein said binding agent comprises a ligand adapted to bind to said cell surface element.
6. A therapeutic composition according to any preceding Claim wherein
15 said binding agent comprises means for ensuring said regulatable agent is internalised into said mononuclear phagocyte.
7. A therapeutic composition according to Claim 6 wherein said means further is adapted to ensure that said therapeutic gene is incorporated into the nucleus of said mononuclear phagocyte.

8. A therapeutic composition according to Claims 1-7 wherein said binding agent is a viral vector.
9. A therapeutic composition according to Claim 8 wherein said vector is an adenoviral vector.
- 5 10. A therapeutic composition according to Claim 8 wherein said vector is a retroviral vector.
11. A therapeutic composition according to Claims 1-6 wherein said binding agent includes mannosylated poly-L lysine.
12. A therapeutic composition according to Claims 3 to 11 wherein said
10 gene encodes a pro-drug activation enzyme.
13. A therapeutic composition according to any preceding Claim wherein said regulatable agent further, or alternatively, comprises a bioreductively activated pro-drug.
14. A therapeutic composition according to any preceding Claim wherein
15 said composition further comprises an agent that activates or controls said regulatable agent.
15. A therapeutic composition according to Claim 14 wherein said agent controls the expression of a gene encoding an activating or control product.
16. A therapeutic composition according to Claims 3 to 15 wherein said

therapeutic gene is under the control of an inducible or repressible promoter element.

17. A therapeutic composition according to Claim 16 wherein said element comprises a selected repressor DNA sequence.

5 18. A therapeutic composition according to Claims 3 or 17 wherein there is further provided a gene encoding a protein that kills mononuclear phagocytes.

10 19. A mononuclear phagocyte that has coupled thereto, or internalised therein, at least a hypoxia and/or ischaemia and/or stress regulatable agent and, optionally, an agent that is adapted to bind to a mononuclear phagocyte ligand which is typically found on the cell surface of said mononuclear phagocyte.

15 20. A method for selectively destroying a mononuclear phagocyte comprising attaching thereto, or internalising therein, a cytotoxic, hypoxically and/or ischaemically and/or stress activated agent; and exposing said mononuclear phagocyte to hypoxic and/or ischaemic and/or stress conditions that occur either artificially by induction or occur/exist naturally.

20 21. A delivery system for targeting therapeutic compositions to hypoxic and/or ischaemic and/or stress sites comprising a hypoxia and/or ischaemia and/or stress regulatable agent and an agent for controlling the functional effectiveness thereof, and coupled thereto, a binding agent for a cell surface molecule of a mononuclear phagocyte.

22. A method for targeting desired agents to hypoxic and/or ischaemic and/or stress sites comprising;

i) coupling at least one of said agents to a binding agent that is adapted for binding or targeting a cell surface molecule expressed by a mononuclear phagocyte;

ii) exposing said coupled agent to a mononuclear phagocyte; and allowing said mononuclear phagocyte to migrate, under conditions that support migration, either on *in vitro* or *in vivo*.

23. A method for treating conditions associated with hypoxic and/or ischaemic and/or stress states comprising administering to an individual to be treated a therapeutic composition according to Claims 1-18.

24. A method for treating conditions associated with hypoxic and/or ischaemic and/or stress states comprising; withdrawing blood and/or serum from an individual to be treated and treating said blood and/or serum *in vitro* with a hypoxically and/or ischaemically and/or stress inducible therapeutic gene under conditions that enable incorporation of said gene into the nucleus of mononuclear phagocytes and re-injecting said treated blood and/or serum into the individual either systemically or directly into a hypoxic and/or ischaemic and/or stress area.

Figure 1

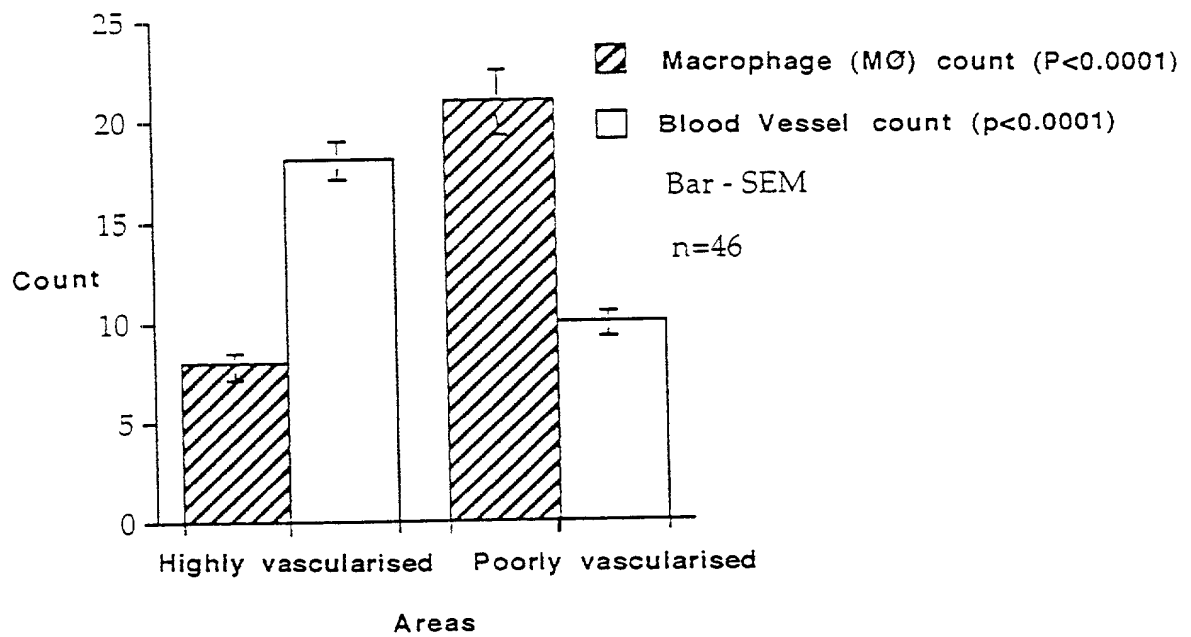


Figure 2

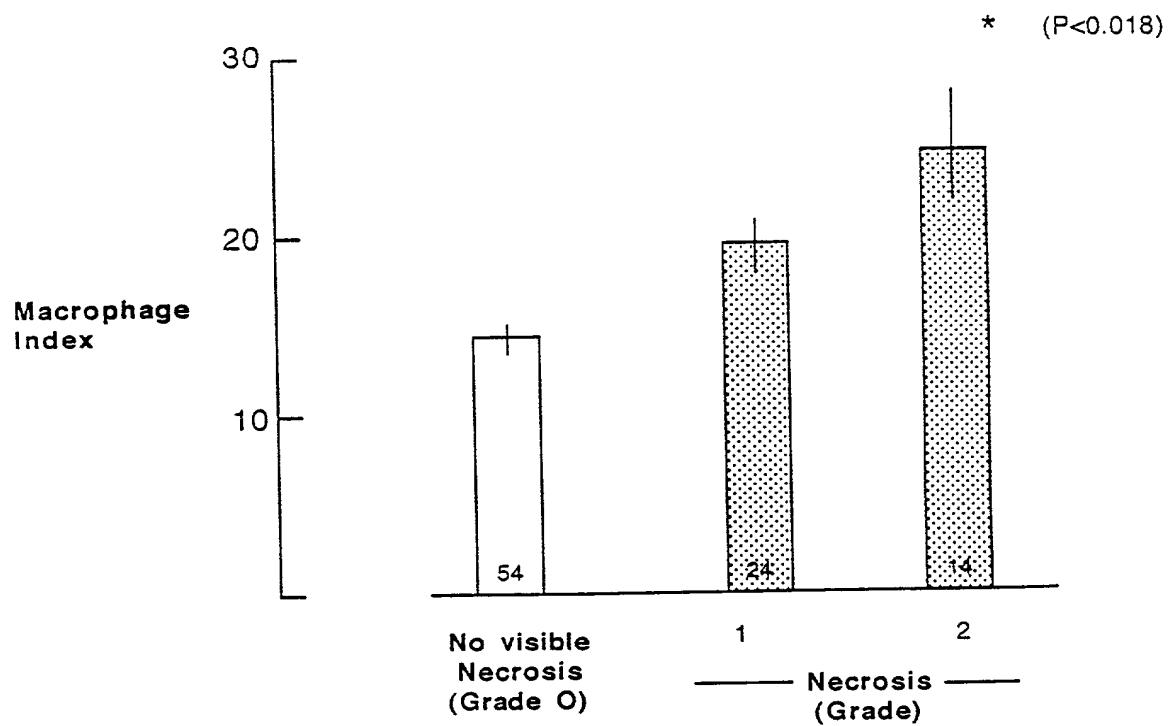
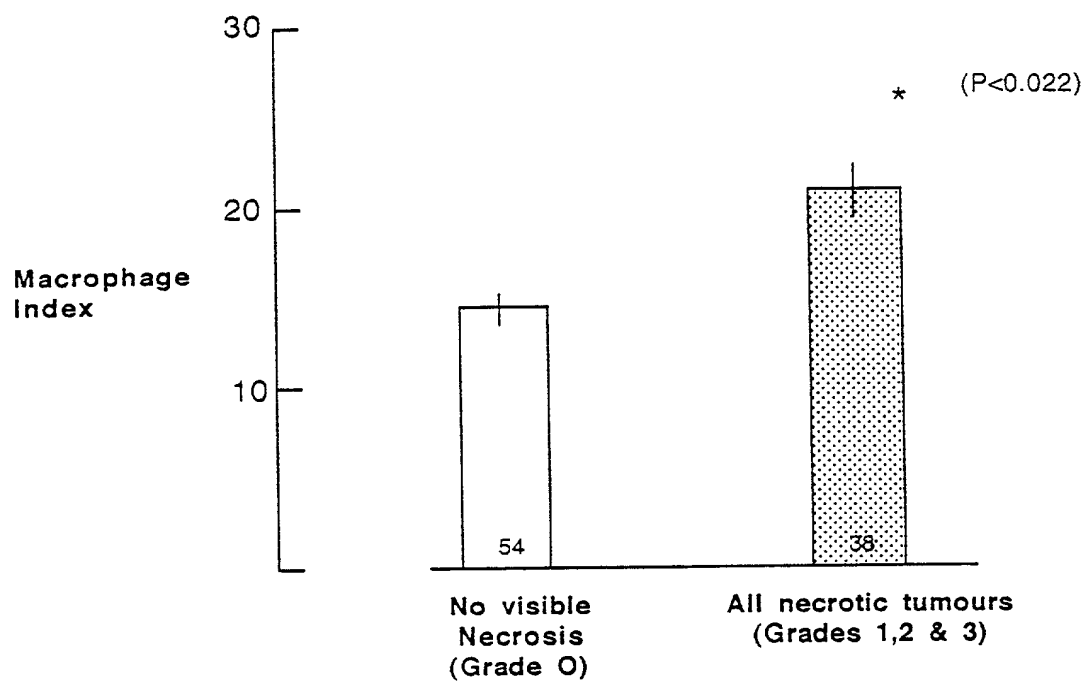


Figure 3

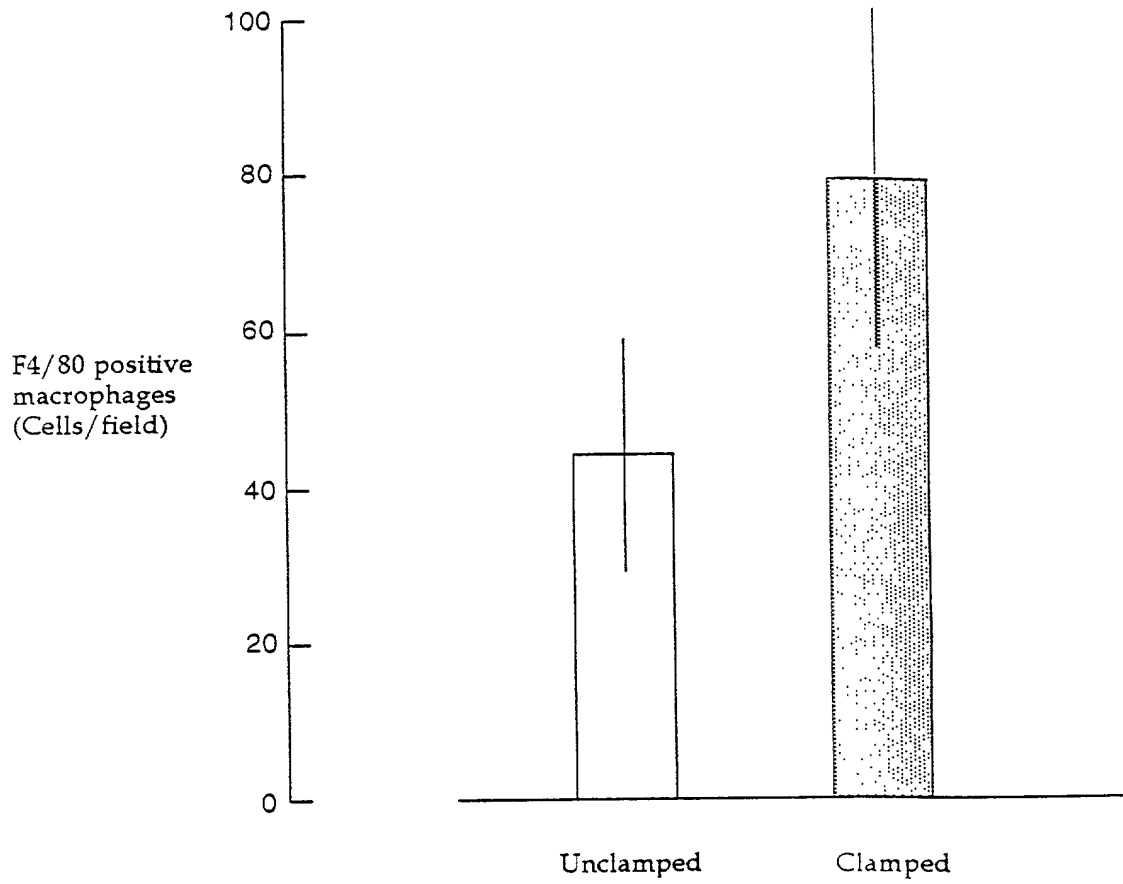


Figure 4

The KpnI-Sall HRE LacZ reporter construct is cloned into the KpnI-XhoI sites of the pADBN transfer vector

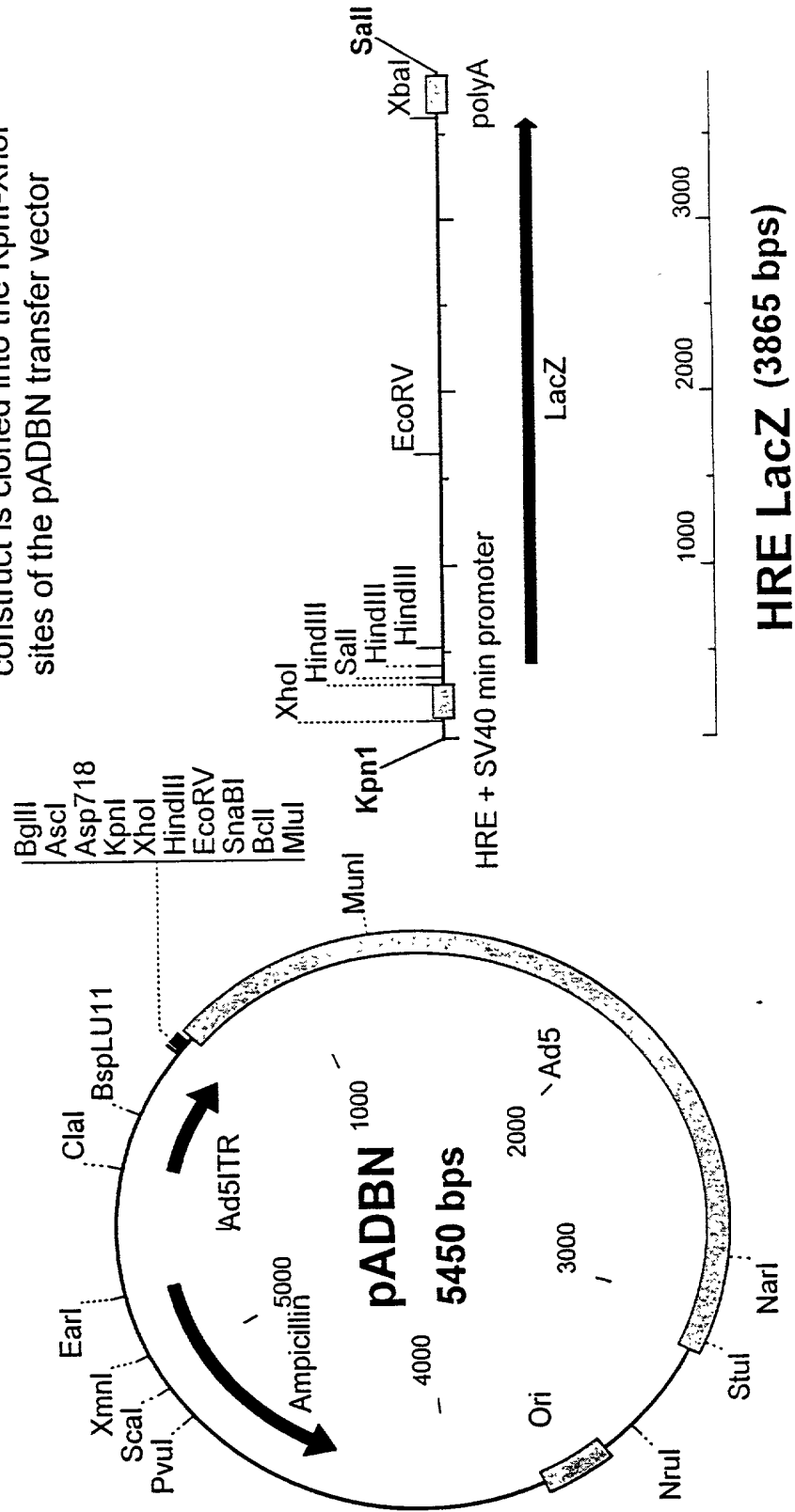


Figure 5(i)

Oxygen tension across
a multicell spheroid
(measured using an
oxygen microelectrode)

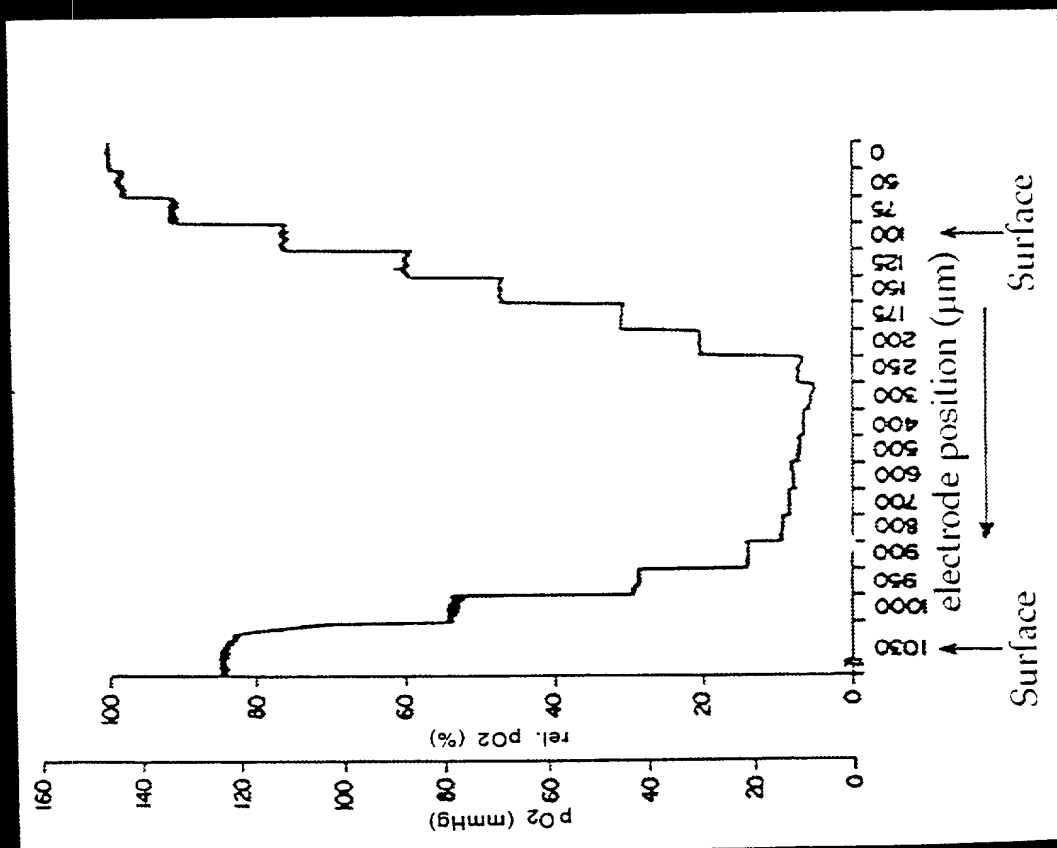
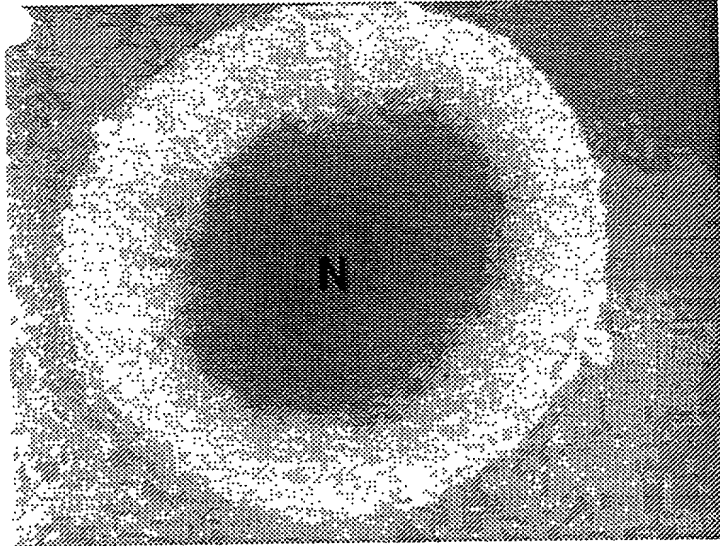


Figure 5 (ii)



Figure 5 (iii)

A



B

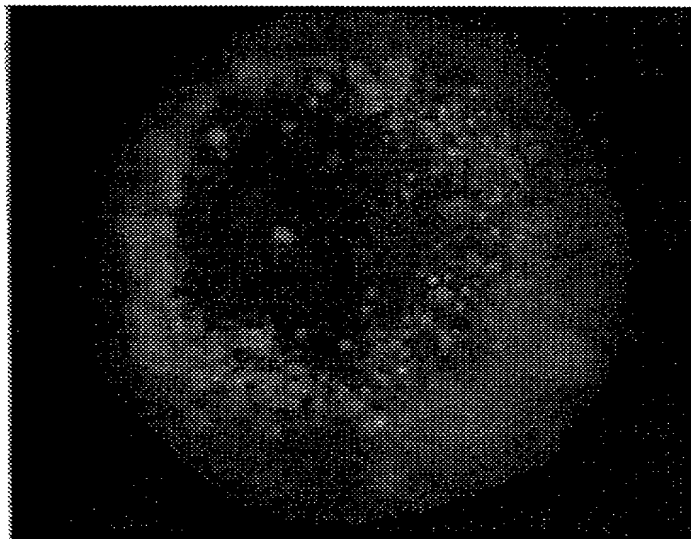
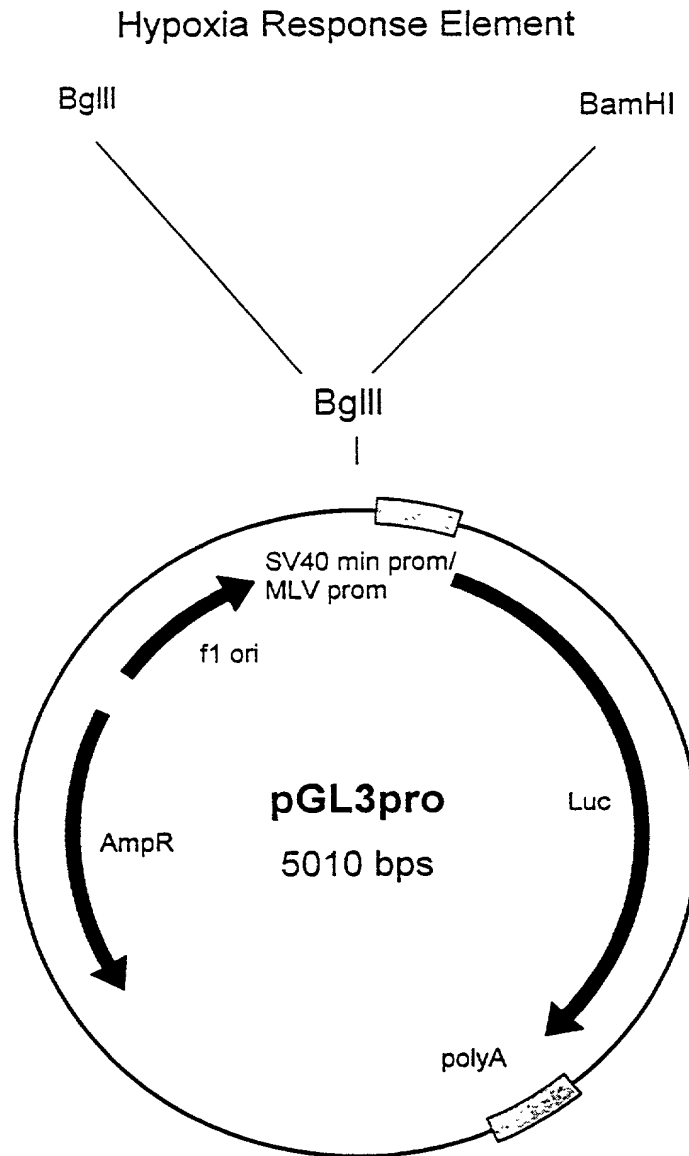


Figure 6

Cloning HREs into Reporter Plasmid



RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MONONUCLEAR PHAGOCYTES IN THERAPEUTIC DRUG DELIVERY

the specification of which (check applicable box(s)):

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. (To Be Assigned) (Atty Dkt. No. 550-128)
☒ was filed as PCT International application No. PCT/GB97/02709 on 8 October 1997
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
9620952.3	Great Britain	9 October 1996
9701975.6	Great Britain	30 January 1997
9703670.1	Great Britain	21 February 1997

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Date/Month/Year Filed
--------------------	-----------------------

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned
PCT/GB97/02709	8 October 1997	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331.*

100 X Inventor's Signature: Claire E Lewis Date: 17/3/99
Inventor: Claire E. Lewis British
(first) (MI) (last) (citizenship)
Residence: (city) Sheffield GB3 (state/country) Great Britain
Post Office Address: The University of Sheffield Medical School, Dept of Pathology, Beech Hill Road, Sheffield, Great Britain
(Zip Code) S10 2RX

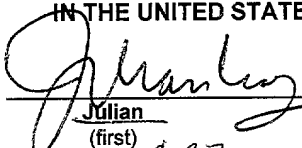
2002 X Inventor's Signature: Adrian L Harris Date: 9/3/99
Inventor: Adrian L. Harris British
(first) (MI) (last) (citizenship)
Residence: (city) Oxford GB3 (state/country) Great Britain
Post Office Address: Isis Innovation Limited, 2 South Parks Road, Oxford, Great Britain
(Zip Code) OX1 3UB

FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet with same information and signature and date for each.

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Page 2

3. ~~Inventor's Signature:~~  Date: 08/03/99
~~Inventor:~~ Julian M Marshall
 (first) (last)
 Residence: (city) Oxford GB3 (state/country) Great Britain
 Post Office Address: Oxford Bioresearch Laboratory, Magdalen Centre, Oxford Science Park, Oxford, Great Britain
 (Zip Code) OX4 4GA
4. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
5. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
6. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
7. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
8. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
9. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
10. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____
11. Inventor's Signature: _____ Date: _____
 Inventor: _____
 (first) MI (last) (citizenship)
 Residence: (city) _____ (state/country) _____
 Post Office Address: _____
 (Zip Code) _____